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Lipase-catalyzed production of novel hydroxylated fatty amides in organic solvent☆

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Abstract

Pseudozyma (*Candida*) *antarctica* lipase B is known to catalyze the direct amidation of carboxylic acids with ammonia in organic solvents. We tested this system for production of primary fatty amides from hydroxy fatty acids including the naturally occurring mono-hydroxy fatty acids, ricinoleic acid (RA) and lesquerolic acid (LQA) and the novel multihydroxy fatty acids, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) and 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD). Reactions were performed at temperatures up to 55 °C. Ricinoleic acid and lesquerolic acid were transformed at initial rates comparable to or better than that of oleic acid, a non-hydroxylated substrate. Transformation percentage at 7 days was better than 95% for all substrates except TOD (93.9%). At 55 °C, most reactions approached completion within 1 day. The primary amides of LQA, DOD, and TOD are novel compounds having melting points of 73, 105, and 100 °C, respectively.

Keywords: Fatty acid amides; Hydroxylated fatty acids; *Candida antarctica* lipase B; Transformation

1. Introduction

There is a great deal of current interest in utilizing renewable raw materials as chemical feedstocks. Oils and fats from plant and animal sources make up a large proportion of current renewable raw material use in the chemical industry [1]. Primary fatty amides, particularly oleamide, erucamide, and stearamide, are important industrial chemicals. They are used in plastics processing as lubricants and as slip and anti-blocking agents [2]. Commercial preparation of primary amides is by the reaction of fatty acids with anhydrous ammonia at high temperature and pressure [3] and therefore enzymatic synthesis under milder conditions has been investigated.

The use of lipase in organic solvent with ammonia as nucleophile has been reported for the production of primary fatty amides. The lipase-catalyzed ammoniolysis of fatty acid esters and triglycerides to form primary amides was initially described by de Zoete et al. [4–6] while Garcia et al. [7,8] reported a similar reaction with β-ketoesters. These reactions were performed with solutions of ammonia in solvent. Litjens et al. [9] used solid ammonium bicarbonate as ammonia source for the ammoniolysis of butyl butyrate to butyramide. The ammoniolysis of esters was used in the above work to avoid precipitation of the ammonium salts of carboxylic acids. However, the work of Litjens et al. [9] indicated that the direct amidation of carboxylic acids with ammonia was feasible. This reaction was subsequently reported for the production of primary amides from a number of carboxylic acids, including oleic acid [10,11]. While five lipases and one esterase were shown to carry out the ammoniolysis of ethyl octanoate to octanamide, *Pseudozyma* (*Candida*) *antarctica* lipase B (CALB) showed the best activity in that reaction [5] and in the direct amidation of oleic acid [10]. Recently, a continuous plug-flow reactor system for the direct

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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amidation of oleic acid to oleamide has been reported [12]. The reactor, which used immobilized CALB (Chirazyme L-2-c.f.C2, Lyo; also known as Novozym 435) catalyzed 85% continuous conversion of oleic acid to oleamide and the authors believed the process to be economically significant.

Hydroxy fatty acids are used in a variety of industrial applications and are useful as chemical intermediates in synthesis reactions [1,13,14]. Mono-hydroxy fatty acids derived from plants include ricinoleic acid [12-hydroxy-*cis*-9-octadecenoic acid], the primary feedstock for the production of nylon-11 [3] and lesquerolic acid [14-hydroxy-*cis*-11-eicosenoic acid]. Ricinoleamide appears in the patent literature as early as 1935 [15], but it was prepared chemically under unfavorable energy conditions. Our laboratory has produced the novel di- and tri-hydroxy fatty acids, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) and 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) via bacterial fermentation of oleic and ricinoleic acids, respectively [16,17]. In this study, we investigated the lipase-catalyzed direct amidation of these hydroxy fatty acids to their corresponding amides. Our aim was to understand and optimize these reaction parameters for subsequent production and characterization of new fatty amides such as those derived from LQA, DOD, and TOD.

2. Materials and methods

Oleic acid [*cis*-9-octadecenoic acid] (OA) and ricinoleic acid were from Nu-Check Prep Inc. (Elysian, MN). Compounds of LQA, DOD, and TOD were produced in the lab as described below. Immobilized *Candida antarctica* lipase B (triacylglycerol acylhydrolase, EC 3.1.1.3) (Novozym 435, CALB), 2-methyl-2-butanol (2M2B, *tert*-amyl alcohol) and ammonium carbamate were purchased from Sigma–Aldrich (St. Louis, MO). Other chemicals were used without further purification.

Lesquerella fendleri oil was a gift from Dr. Terry Isbell, New Crops and Processing Technology Research Unit, NCAUR, USDA-ARS, Peoria, Illinois. Crude LQA was prepared by a basic saponification procedure. Briefly, lesquerella oil (200 mL) was refluxed with 84 g KOH in 150 mL methanol for about 3 h. Two batches of saponification were carried out, combined, and then adjusted to pH 4–5 with chilled 4 M HCl. The saponified mixture was extracted with an equal volume of hexane. Thereafter, the solvent-extracted mixture was washed with water in a separatory funnel until the pH became neutral. The free fatty acids from *L. fendleri* were subsequently washed with sodium phosphate buffer (117 g/L NaH_2PO_4 and 10 g/L Na_2HPO_4, pH 5), dried over anhydrous sodium sulfate and concentrated by a rotary evaporator at 40 °C. Crystallization of LQA (about 25 g) was achieved from 1 L hexane upon sequential cooling at 5, −10, and −20 °C for 24 h at each temperature. The crystals were quickly collected on a 1PS Whatman filter paper under freezing (−20 °C) conditions and transferred to a container. The crude acid was flushed with an ultra pure grade of nitrogen gas and stored in batches at below 0 °C. The procedure produced 150 g LQA from 400 mL *Lesquerella fendleri* oil. The LQA preparation was about 98% pure based on GC analyses.

Microbial conversion of oleic acid in a bioreactor process was used to obtain crystalline DOD [18], while microbial conversion of ricinoleic acid was used to obtain crystalline TOD [19].

Amidation reactions consisted of 15 mL of 2M2B containing 100 mM fatty acid, an appropriate amount of ammonium carbamate, and 150 mg (10 mg/mL) immobilized enzyme. Ammonium carbamate and the immobilized enzyme were stored at 4 °C over calcium sulfate (Drierite) and the solvent was dried over anhydrous sodium sulfate prior to use. The mixtures were equilibrated at temperature overnight prior to addition of enzyme to start the reaction. Transformation reactions were performed in triplicate in septum-sealed 16 mm × 125 mm screw-cap test tubes. Negative controls consisted of reaction mixtures without added enzyme. Agitation was provided by a Labquake test-tube rotator (Barnstead Intl., Dubuque, IA). Samples were taken through the septa by syringe.

Oleic acid, RA, and LQA transformation was determined by GC. Fatty acids were converted to methyl esters with diazomethane prior to analysis with an HP 5890 Series II gas chromatograph equipped with a model 18593B autosampler, HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness) and an FID detector. Analytical conditions were as follows: injector, 200 °C; detector, 290 °C; initial oven temperature 200 °C for 1 min ramping to 270 °C at 10 °C/min and holding for 10 min. One microliter of a tetrahydrofuran solution was injected in split mode.

DOD and TOD transformation was determined on a Shimadzu HPLC system consisting of two pumps, a column oven, autosampler, diode-array detector and system controller. Samples (5 μL in methanol) were run isocratically with a mobile-phase of methanol:water (60:40) containing 0.1% acetic acid on a Varian Polaris C8-A column (150 mm × 4.6 mm) at 40 °C. Peaks were monitored at 201 nm.

Experiments were ended by filtering out the immobilized enzyme. Products were recovered by crystallization in ethyl acetate after removing the reaction solvent by vacuum evaporation. Recovered product purity was estimated by chromatogram peak area percentages on GC and HPLC.

3. Results and discussion

Immobilized CALB (Novozym 435) is thermostable and can be used at 60–80 °C for long periods of time without loss of activity [20]. CALB-catalyzed ester ammoniolysis and the direct amidation of fatty acids with ammonia have been carried out in a number of solvents, including *tert*-butanol, 2M2B, methyl isobutyl ketone (MIBK), and dioxane [5,7,9,10,12]. Slotema et al. [12] compared four solvents for

the direct amidation of oleic acid to oleamide and determined that 2M2B provided the best combination of initial reaction rate and equilibrium conversion. High initial concentrations of ammonia may cause the precipitation of ammonium salts of the fatty acid substrate, depending on the solvent and temperature, and result in a reduced initial reaction rate [10]. For this reason, Litjens found that adding ammonia as a solid (ammonium bicarbonate or ammonium carbamate) and allowing it to slowly dissolve as the reaction progressed gave higher initial reaction rates than using ammonia gas. Water is formed as a product of the reaction and increasing the initial water concentration in the reaction mix results in lower equilibrium transformation and initial reaction rates [12], therefore ammonium carbamate is preferred as an ammonia source because it does not form water as it dissolves. Given these results, we selected 2M2B as solvent and ammonium carbamate as ammonia source for our work.

Transformations of OA, RA and LQA were performed at four temperatures (25, 35, 45 and 55 °C) while DOD and TOD transformations were done at 55 °C. The ammonia:fatty acid ratio for these experiments was 2:1 (100 mM ammonium carbamate giving 200 mM ammonia). This concentration of ammonium carbamate was completely soluble in 2M2B at 55 °C, but not at the lower temperatures. Transformations were monitored as the utilization of the fatty acid substrate with the initial transformation rate being the inverse of the utilization rate and expressed as μmol fatty acid transformed min^{-1} g $enzyme^{-1}$, while the percent transformed is the inverse of the percentage of the initial fatty acid substrate utilized. For each substrate there was only one product peak evident on chromatograms and it was assumed that all substrate utilized was transformed into the amide product. Purified products from the OA, RA and LQA reactions were analyzed by GC–MS. Authentic oleamide (Sigma–Aldrich, St. Louis, MO) and the oleic acid reaction product were both identified by a mass spectral library (Wiley Registry of Mass Spectral Data, 7th edition, John Wiley and Sons, Hoboken, NJ) as 9(Z)-octadecenamide (oleamide) while the mass spectra of the RA and LQA products indicated the presence of the characteristic primary amide.

The results of the 25 and 55 °C experiments are shown in Fig. 1. Lesquerolic acid transformation was the most rapid of the substrates tested with completion of the reaction being approached at 3 days even at the lowest temperature. OA and RA were transformed at similar rates and required 7 days to approach completion at 25 °C. The initial rate of transformation versus temperature is shown in Fig. 2. The initial rate of LQA transformation at 55 °C was 26.9 μmol min^{-1} g $enzyme^{-1}$ while the rates of OA and RA were approximately 18 μmol min^{-1} g $enzyme^{-1}$. DOD and TOD rates were 4.9 and 14.4 μmol min^{-1} g $enzyme^{-1}$, respectively. An Arrhenius plot of the data (Fig. 3) resulted in calculated activation energies of 50, 47.6, and 32.4 kJ/mol for OA, RA, and LQA, respectively.

The higher rate of LQA, a C-20 fatty acid, versus RA and OA (C-18) is interesting. CALB has been shown to have a

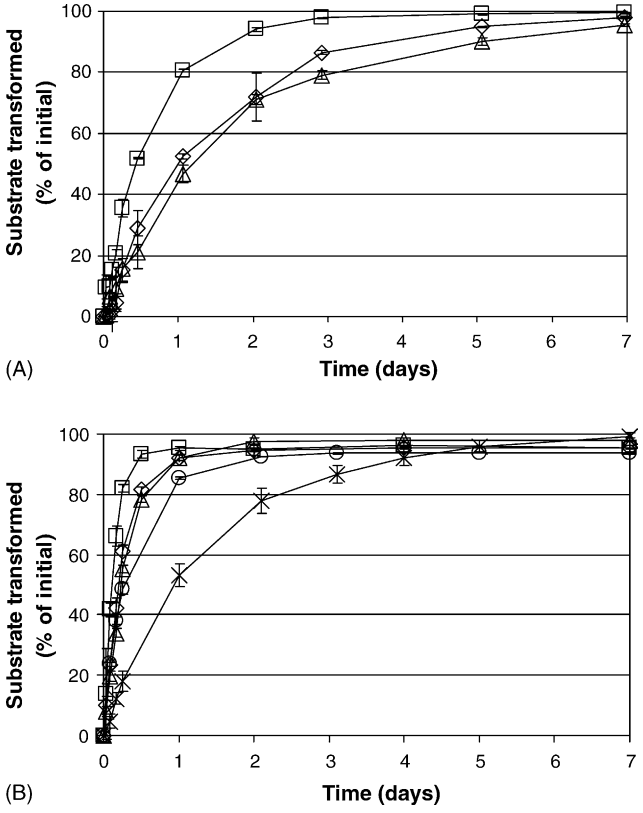

Fig. 1. Fatty acid transformation at (A) 25 °C and (B) 55 °C. LQA, square; RA, diamond; OA, triangle; TOD, circle; DOD, cross. Data points are the average of three replicates ±1 S.D.

preference for shorter chain (C-6 to C-10) fatty acids in esterification reactions, although acids above C-18 were not tested and this preference was reduced somewhat when the immobilized enzyme (Novozym 435) was used [21–24]. Pleiss et al. [25] determined that fatty acids up to C-13 in length fit into the CALB binding site which may account for the preferential enzyme activity towards short and medium-chain fatty acids. In a study of triglyceride hydrolysis, CALB showed no preference between unsaturated fatty acids with chain-lengths from C-18 to C-24 [26].

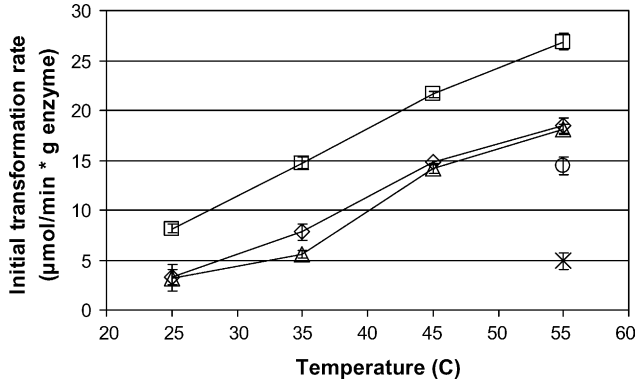

Fig. 2. Initial rate of transformation at different temperatures. LQA, square; RA, diamond; OA, triangle; TOD, circle; DOD, cross. Data points are the average of three replicates ±1 S.D.

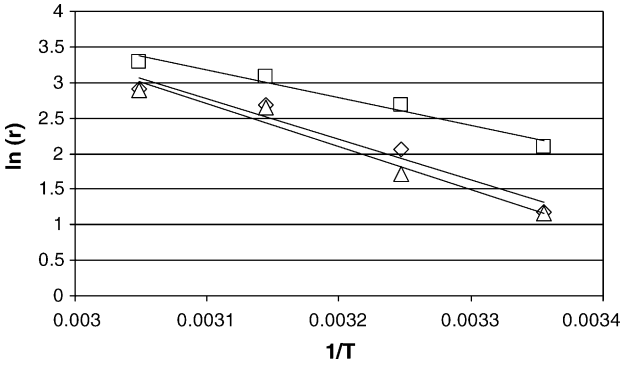

Fig. 3. Arrhenius plot of initial rate (r) vs. temperature. LQA, square; RA, diamond; OA, triangle. Data points are the average of three replicates.

The rate of RA transformation consistently was slightly higher than that of the unhydroxylated OA (Figs. 1 and 2). The structural study of the binding site of CALB [25] showed a flat, hydrophobic region in the area of binding of the C-7 to C-13 carbons. Apparently, hydroxylation at C-12 does not interfere with binding and may help slightly. It is interesting to note that while DOD, hydroxylated at the 7 and 10 carbons, had a significantly reduced transformation rate; addition of a hydroxyl at the 12 carbon (TOD) ameliorated this effect to a degree. It is not known what effects the shift in unsaturation from *cis*-9 to *trans*-8 configuration (DOD and TOD) had on the reaction rate. CALB has been shown to exhibit a slight preference for eliadic acid (*trans*-9-octadecenoic acid) compared to oleic acid [27].

For the three fatty acids tested at different temperatures, all reactions approached completion within 1 day at the two higher temperatures (Fig. 4). One-day transformation percentage at 25 and 35 °C was the lowest for OA (47 and 62%, respectively) followed by RA (53 and 72%, respectively) and LQA (81 and 91%, respectively). Excluding the 25 °C data for which a stable endpoint had not yet been reached at 7 days for OA and RA, the average endpoint transformation percentage was 98.4 for OA (range 99.1–97.9); 95.8 for RA (range 97.1–95.0); and 96.3 LQA (range 98.7–95.0). The 7-day transformation of DOD and TOD at 55 °C was 99.0 and 93.9%, respectively (Fig. 1).

Experiments were also performed to determine the effect of ammonia concentration on initial rate and endpoint transformation. They were carried out at 55 °C with ammonia:fatty acid ratios of 1:1, 2:1, and 3:1 (100 mM fatty acid). The concentrations of ammonium carbamate used were completely soluble at this temperature. Increasing the ratio of ammonia to fatty acid from 2:1 to 3:1 resulted in an increase in the initial reaction rate for both RA and LQA (Fig. 5). The RA transformation rate (μmol min^{-1} g enzyme^{-1}) increased to 25 from 18.5 (35%) while that of LQA increased to 35.7 from 26.9 (33%). The increase in the OA rate from a substrate ratio of 2:1 to 3:1 and the differences of the initial rates for all three fatty acids between the 1:1 and 2:1 substrate ratios were small. A drop in the substrate ratio from 2:1 to 1:1 resulted in a drop in transformation to approximately 80% (OA, 81.1%; RA, 80.6%; LQA, 80.4%). The OA rate of 17.1 μmol min^{-1} g enzyme^{-1} and transformation of 81% at the 1:1 ratio are in close agreement with the values of 20 μmol min^{-1} g enzyme^{-1} and 76% derived by Slotema et al. [12] for the same concentrations (at 60 °C).

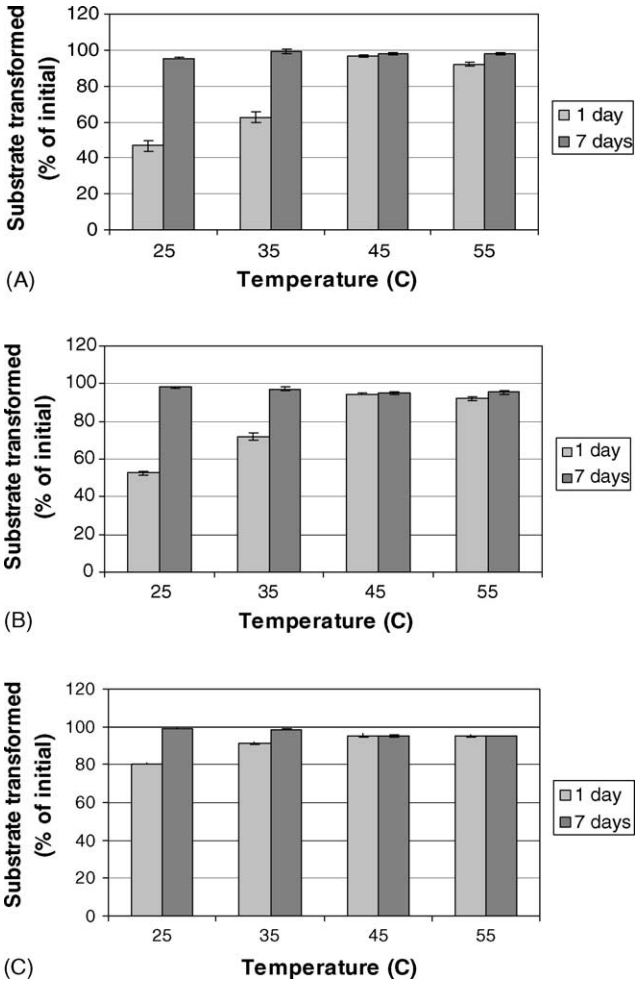

Fig. 4. Percentage of substrate transformed at 1 and 7 days: (A) oleic acid; (B) ricinoleic acid; (C) lesquerolic acid. Bars are the average of three replicates ±1 S.D.

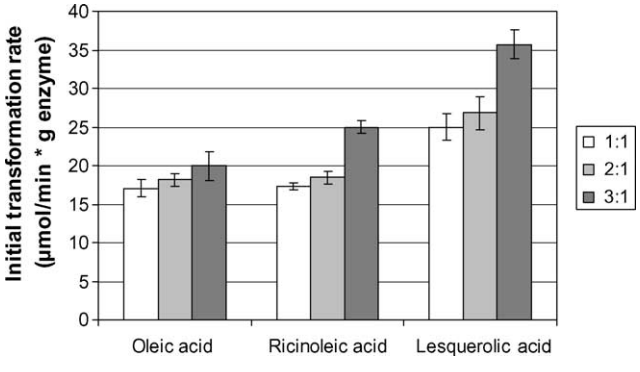

Fig. 5. Initial transformation rate at 55 °C with different ammonia to fatty acids substrate ratios (ammonia:fatty acid). Rates are the average of three replicates ±1 S.D.

Table 1
Melting points (°C) of fatty acids and their corresponding fatty amides

	Oleic	Ricinoleic	Lesquerolic	DOD	TOD
Acid	13.4[a]	5.5[a]	N.D.	63.5–64	94.5–95
Amide	75	67	73	105	100

N.D.: not determined; DOD: 7,10-dihydroxy-8(*E*)-octadencenoic acid; TOD: 7,10,12-trihydroxy-8(*E*)-octadencenoic acid.
[a] From literature.

The purified amides exhibited a range of melting points (Table 1). Ricinoleamide had the lowest melting point at 67 °C while the amide of DOD was the highest at 105 °C. Enzyme-prepared fatty amides all exhibited melting points higher than their fatty acid counterparts, a property that could make them useful in a variety of applications.

4. Summary

An immobilized lipase (CALB, Novozym 435) catalyzed the direct amidation of several hydroxy fatty acids to their primary amides with ammonia in organic solvent. Lesquerolic acid was transformed at the highest initial rate while ricinoleic acid transformation was nearly identical to that of oleic acid. The initial reaction rates of the di- and tri-hydroxy fatty acids (DOD and TOD) were below those of the other substrates, with the DOD rate being the lowest. At 55 °C and a 2:1 ammonia to fatty acid substrate ratio, the transformation reactions of LQA, RA, and OA approached completion within 1 day. Endpoint transformation for these conditions was better than 95% for all substrates except TOD (93.9%). Increasing the ammonia to fatty acid ratio to 3:1 at 55 °C resulted in increased initial reaction rates for the substrates tested while decreasing the ratio to 1:1 reduced the transformation to approximately 80%. The amides of LQA, DOD, and TOD are novel compounds and exhibited melting points of 73, 105, and 100 °C, respectively. Therefore, we have succeeded in the enzymatic preparation of novel hydroxy fatty amides for further tests for potential new uses. This is the first report on the enzymatic preparation of novel hydroxy fatty amides in higher yields.

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